Frequent ESR1 and CDK Pathway Copy-Number Alterations in Metastatic Breast Cancer

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Abstract

DNA sequencing has identified a limited number of driver mutations in metastatic breast cancer beyond single base-pair mutations in the estrogen receptor (ESR1). However, our previous studies and others have observed that structural variants, such as ESR1 fusions, may also play a role. Therefore, we expanded upon these observations by performing a comprehensive and highly sensitive characterization of copy-number (CN) alterations in a large clinical cohort of metastatic specimens. NanoString DNA hybridization was utilized to measure CN gains, amplifications, and deletions of 67 genes in 108 breast cancer metastases, and in 26 cases, the patient-matched primary tumor. For ESR1, a copyshift algorithm was applied to identify CN imbalances at exon-specific resolution and queried large data sets (>15,000 tumors) that had previously undergone next-generation sequencing (NGS). Interestingly, a subset of ER+ tumors showed increased ESR1 CN (11/82, 13%); three had CN amplifications (4%) and eight had gains (10%). Increased ESR1 CN was enriched in metastatic specimens versus primary tumors, and this was orthogonally confirmed in a large NGS data set. ESR1-amplified tumors showed a site-specific enrichment for bone metastases and worse outcomes than nonamplified tumors. No ESR1 CN amplifications and only one gain was identified in ER+ tumors. ESR1 copyshift was present in 5 of the 11 ESR1-amplified tumors. Other frequent amplifications included ERBB2, GRB7, and cell-cycle pathway members CCND1 and CDK4/6, which showed mutually exclusivity with deletions of CDKN2A, CDKN2B, and CDKN1B.

Implications: Copy-number alterations of ESR1 and key CDK pathway genes are frequent in metastatic breast cancers, and their clinical relevance should be tested further.

Introduction

Breast cancer is a genetic disease driven by accumulations of single-nucleotide mutations and structural alterations (1). The latter include gene fusions, deletions, tandem duplications, and copy-number (CN) amplifications as seen in ERBB2 (HER2), CCND1, and MYC. Numerous studies have shown varying levels of estrogen receptor (ERalpha) gene (ESR1) CN amplifications in primary breast cancer. In 2007, Holst and colleagues used fluorescence in situ hybridization (FISH) and identified ESR1 CN amplifications (20.6%) and gains (15.3%) in primary breast tumors (2). Subsequently, multiple groups reported ESR1 CN amplifications at much lower frequency (0%–10%), using FISH and array comparative genomic hybridization (aCGH; refs. 3–8). Analysis of The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) data sets using cbioPortal (9–11) revealed that ESR1 CN amplifications are present in only 2.5% and 2.3% of breast cancer samples heavily enriched for primary disease, respectively. More recently, Desmedt and colleagues showed ESR1 CN gains and corresponding increased ESR1 mRNA levels in 25% of primary invasive lobular breast cancers (ILC; ref. 12). Thus, the true frequency as well as the relevance of these ESR1 CNV events, particularly in recurrent and metastatic disease, has yet to be determined.
The landscape of structural variants in metastatic breast cancer (MBC) is largely unknown, particularly after therapy resistance. Gene rearrangements and CN amplifications of ESR1, FOXA1, CYP19A1, and ESRB2 have been reported in relatively small cohorts of endocrine-resistant breast cancers (13–16). Li and colleagues (17) originally described an ESR1 fusion arising in a patient-derived xenograft (PDX) model, and we have recently identified nine additional ESR1 fusions in advanced disease (18). Importantly, the location between the N-terminal and the C-terminal fusion partner is often conserved between ESR1 exons 6 and 7. Gene fusion events are often associated with CN alterations adjacent to the fusion junction that results in an imbalance of DNA CN between the breakpoint flanking exons (19, 20). We therefore designed and applied a computational method (copyshift) that identifies CN imbalances and found imbalances were significantly enriched in ER+ metastases compared with primary breast tumors (18).

Despite overwhelming evidence for a critical role of ESR1 mutations and increasing evidence for ESR1 fusions in endocrine-resistant breast cancer, a comprehensive study of ESR1 CN variations in recurrent and metastatic disease is lacking. In this study, we set out to characterize ESR1 CN alterations with exon-level resolution in a large cohort of well-curated metastatic lesions using a highly sensitive nanoString-based approach. Additionally, we assessed the CN alteration landscape of 66 genes with known roles in breast cancer progression and therapy resistance.

Materials and Methods

Sample collection

We obtained 141 formalin-fixed paraffin-embedded (FFPE) and frozen sections from eligible metastatic cases from the University of Pittsburgh Health Sciences Tissue Bank (HSTB) and Charité Universität medizin, Berlin. Collection and analysis of specimens was approved under the University of Pittsburgh IRB and Charité Universität medizin Berlin guidelines. When available, patient-matched treatment-naïve primary tumors were included in the study (n = 26). Seven samples were excluded from the study due to unknown ER status. In total, we had 134 samples from 100 patients who met the study criteria (82 ER+ and 52 ER-). Including 26 primary tumors (15 ER+ and 11 ER-) and 108 metastases (67 ER+ and 41 ER-). In some cases, multiple primary tumors or metastasis were from the same patient. More than 85% of the samples had tumor cellularity > 40%, which was determined by surface area of tumor cells. If it was lower than 30% to 40%, we macrodissected the tumor and used. Therefore, we circled an area of interest on the slide (i.e., a subregion of the slide that contains the highest concentration of invasive cancer), and from within this region, we estimated the percentage of nuclei that are tumor cell nuclei.

Clinicopathologic characteristics are provided in Additional file 1: Supplementary Table S1. We collected information on ER status, source of tissue, site of metastasis, and histologic subtype. A subset of the samples has recently been used in another, unrelated study (ref. 21; indicated in Supplementary Fig. S1).

Immunohistochemistry (IHC)

Hematoxylin and eosin (H&E) staining procedures were performed at the Histology and Micro-imaging Core (HMC) facility at Magee-Women’s Research Institute (MWRI). Analysis of histologic classification was performed by two pathologists (P.C. Lucas and E. Elishaev; Additional file 1: Supplementary Table S1).

DNA isolation

DNA was isolated from 4 to 6 FFPE macrodissected sections (10 μm) per sample depending on tumor size and cellularity using the Qiagen AllPrep FFPE kit (cat. #80234) as per the manufacturer’s instructions. DNA from frozen samples was extracted using the Qiagen DNAeasy kit (cat #69506) according to the manufacturer’s instructions. All DNA quantifications were done using Qubit dsDNA HS/BR assay kits (Thermo Fisher).

Control samples for nanoString and digital droplet PCR (ddPCR) comparison

We isolated DNA from MCF-7 long-term estrogen-deprived (LTED) cells, BT-474, and MCF10A cells for validation of CN calls. MCF-7 LTED cells have ESR1 amplifications (17) and BT-474 cells have been described to have a heterozygous ESR1 deletion [Cancer Cell Line Encyclopedia (CCLE); ref. 22]. MCF10A is a nontumorigenic epithelial cell line with normal (2N) ESR1 CN (23).

Digital droplet PCR (ddPCR)

Primers and probes were designed and ordered through Integrated DNA Technologies (IDT) for ESR1 and Bio-Rad for two reference genes recommended by the manufacturer (EIF2C and AP3B1; Additional file 1: Supplementary Table S2). Control samples (60 ng) were processed for ddPCR analysis as previously described (24). Briefly, DNA samples were combined with primers, probes, and supermix, and then added to cartridge. Droplets were generated using Bio-Rad QX100 Droplet Generator and transferred into a 96-well plate for PCR amplification, and droplet reader (Bio-Rad QX100 Droplet Reader) was used to count PCR+ and PCR- droplets. Data were analyzed using QuantaSoft software (Bio-Rad) where target concentration was normalized to reference concentration and multiplied by the number of reference loci in the genome (assumed to be 2) to generate CN calls.

nanoString

We designed 100 bp DNA hybridization probes for a total of 67 genes (Additional file 1: Supplementary Table S3), including ESR1 (n = 10 probes; Additional file 2: Supplementary Fig. S1), and 66 genes with known roles in breast cancer development and progression (n = 3 probes per gene). For the latter, we queried TCGA breast cancer data set and previous breast cancer metastasis studies (25–28), with a focus on potentially druggable genes as identified in drug gene interaction database (DGIdb; ref. 29). Processing of CN data was performed as per manufacturer recommendations. Briefly, DNA samples were fragmented at 37°C using Alu1 restriction enzyme, denatured at 95°C, and hybridized overnight with target probes. Posthybridization sample processing was done using the automated nCounter Prep Station. Raw counts were then collected from the nCounter Digital Analyzer and transferred to the nSolver software (v 2.5) for data analysis (30). Raw counts were normalized to 10 invariant reference probes (intrasesamle count normalization) and to reference sample pools of normal breast FFPE (n = 13) and frozen (n = 4) DNA to generate CN calls (intersample normalization; Additional file 1: Supplementary Tables S3–S5). Average CN estimate value was calculated per gene based on all probes for that gene relative to the CN estimate in the normal sample pool. We used the 67 genes of the nanoString code set for comprehensive quality control assessment of CN calls between FFPE and cell pellet DNA.
Preparation and sequencing of RNA-seq libraries

RNA-seq data were available for a subset of 66 samples (46 ER⁺) and was used to associate correlation between ESR1 CN and mRNA expression. TruSeq RNA Access library preparation (Illumina) and sequencing was performed at the sequencing core facility at Children's Hospital of University of Pittsburgh Medical Center (UPMC) using the NextSeq500 platform that produced paired-end reads (2 x 75 bp). Gene-expression values for ESR1 were calculated using counts per million normalized to trimmed-means of M-values (TMM-normalized count per million (CPM)) using DESeq2 and edgeR packages in R (31, 32).
Figure 2.
Frequent ESR1 amplifications in MBC. A, Oncoprint visualization of ESR1 CN alterations in ER⁺ (top) and ER⁻ (bottom) samples. Levels of amplification and deletion are color coded. Each column represents a single sample (sample IDs labeled as gray; primary tumor, black; metastasis). Each row indicates CN call of the correspondent single exon probe. Untranslated exons (E1 and E2) are annotated with "/C3" symbol. P, promoter probe. ESR1_ave: average CN call of all probes. B, Distribution of ESR1 CN amplifications by site (primary, brain, bone, GI, and ovaries as indicated by arrows) and ER status (color coded). Bone metastases showed significant enrichment for amplifications versus primary and ovaries (Fisher exact test, \( P < 0.05 \)). C, Single pair where the brain metastasis had higher level ESR1 amplifications compared with its primary tumor. D, Graphical representation (left; actual copy number) and heat map (right; ratio) of the 50–30 CN imbalance among the ESR1-amplified samples. The dark red color indicates higher CN amplification toward the 5' side of ESR1 (Wilcoxon matched-pairs signed-rank test \( P = 0.0024 \)).
Genomic CN analysis of RNA-seq data

Determination of chromosomal aberrations was based on measuring allelic bias in RNA-seq data using the package eSNP-Karyotyping in R (33). Briefly, we first called variants using GATK package Haplotype caller (34). Variants were then ordered according to their genomic coordinates and the ratio between major and minor alleles was calculated. To increase confidence, variants with coverage below 20 reads and frequency below 0.2 were excluded from the analysis. Statistical significance was calculated with a one-tailed t test comparing the SNP’s major/minor ratio values in each window with the total SNP pool and false discovery rate (FDR) correcting for multiple testing. Moving median plots were generated using windows of 151 SNP’s as suggested by the package developers.

ESR1 CN validation data sets

Comprehensive genomic profiling was performed on breast tumors in a CLIA-certified, New York State– and CAP-accredited laboratory [Foundation Medicine (FM); refs. 35, 36]. In addition, tumor data from the American Association for Cancer Research (AACR) GENIE and Memorial Sloan Kettering Cancer Center (MSKCC) were accessed and analyzed through cBioPortal (37, 38). Statistical analyses with P value significance of 0.05 were performed in R and GraphPad, and figures were plotted using ggplot2.

Statistical and bioinformatics analysis

A environment was used for statistical computing and graphics (39). Oncoprints visualizing multiple genomic alterations were generated using ComplexHeatmap package (40). Given the important role of ESR1 in breast cancer development and progression, and prior evidence for low-level amplifications, CN changes of ESR1 were a major focus of our study. Thus, we used 10 probes to cover the ESR1, and only 2 to 3 probes for the other 66 genes. ESR1 CN increase by 35% (CN ≥ 2.7) and decrease by 50% (CN ≤ 1) were considered gains/amplifications and deletions, respectively. CN calls above ≥10 were considered high amplifications. Giving the lower resolution for other genes in the panel, we used one CN cutoff of ≥5 as amplification. Plots and heat map of actual CN imbalance and shifts were generated using ggplot2 and heatmap.3 packages, respectively (41, 42). For CN imbalance, we reported only shifts of ≥30% difference in CN (ratio of ≥1.3) between ESR1 3’ and 3’ exons, which is similar to the 35% CN increase used to define gains. Multiple correlations between the different ESR1 exons and mRNA expression were clustered by first principal component (FPC) scores. Spearman and Pearson correlations, Fisher exact, and Wilcoxon rank-sum tests were performed in R and GraphPad Prism with significance cutoff of 0.05.

Results

Measurement of DNA CN changes using nanoString technology

We set out to determine which platform (ddPCR or nanoString) is best suited for quantifying CN alterations. Using ddPCR, we determined ESR1 CN status in MCF7-LTED, BT-474, and MCF10A cells, with EIF2C2 and AP3BI reference probes. Our CN calls were consistent between the two probes for the ESR1 CN normal (MCF10A) and deletion (BT-474) models, but not MCF7-LTED cells, that have a known ESR1 amplification (Fig. 1A and Additional file 1: Supplementary Table S6). These data suggested that use of two reference probes was not sufficient for accurate measurement of CN. Next, we measured ESR1 CN using nanoString technology, where 10 reference probes were included in the library. This analysis correctly identified CN in the three control cell lines (Fig. 1C). We then performed quality control experiments to test sensitivity and reproducibility of the CN calls within and between the nanoString runs using DNA from fresh cell pellets and from processed FFPE sections. There was a high correlation between DNA isolated from fresh and fixed samples (Fig. 1B and C, and Additional file 1: Supplementary Table S8; rho > 0.9, P < 2.2e−16) and excellent reproducibility between different nanoString runs using the same DNA (Fig. 1D and E; Supplementary Table S9; rho > 0.99, P < 2.2e−16). We therefore proceeded using nanoString technology for the characterization of CN alterations in a large set of well-curated breast tumors.

Frequent ESR1 amplifications in MBC

Our probe design targeted the ESR1 promoter region, untranslated exons (E1 and E2), and coding exons (E3–E10; Additional file 2: Supplementary Fig. S1). Given the high resolution of our coverage, we were able to call low-level amplifications/gain (CN ≥ 2.7), in addition to amplification (CN ≥ 10). We measured ESR1 CN in a total of 134 tumor samples (82 ER+ and 52 ER−; Fig. 2). When averaging CN calls from all ESR1 probes, we detected ESR1 CN alterations in 11 (13.4%) out of 82 ER+ tumor samples (67 metastases and 15 primary; Fig. 2A; Table 1). Specifically, eight (9.8%) samples had CN gains and three (3.7%) samples had amplifications. There was a trend for enrichment of ESR1 CN alterations in metastatic samples (14.9%) compared with primary samples (6.6%). Bone metastases showed significant enrichment for ESR1 CN alterations versus primary breast cancer and ovarian metastases (Fig. 2B; Table 1; P < 0.05).

In ER+ tumors (41 metastases and 11 primary), we did not detect any ESR1 amplifications and found only one primary tumor with a CN gain (Fig. 2A and Additional file 1: Supplementary Table S10). ESR1 CN alterations were significantly enriched in ER+ versus ER− tumors (Table 2; P = 0.0192).

Table 1. ESR1 CN alterations by site in ER+ samples

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>AMP</th>
<th>Gain (%)</th>
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<tr>
<td>Primary</td>
<td>15</td>
<td>0.0</td>
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<tr>
<td>Bone</td>
<td>21</td>
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<td>1 (4.8)</td>
</tr>
<tr>
<td>Bone</td>
<td>18</td>
<td>2 (11.1)</td>
<td>5 (27.8)</td>
</tr>
<tr>
<td>Ovaries</td>
<td>22</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>GI</td>
<td>6</td>
<td>0.0</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td>Total</td>
<td>82</td>
<td>3 (3.7)</td>
<td>8 (9.8)</td>
</tr>
</tbody>
</table>

*Significant enrichment for amplifications versus primaries and ovaries (Fisher exact test, P < 0.05).

N = number of samples included in the cohort; n and (%) = count and percentage of samples with alterations for the indicated site, respectively.

Table 2. ESR1 CN alterations by ER status

<table>
<thead>
<tr>
<th>ER status</th>
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<th>AMP</th>
<th>Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER+</td>
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<td>3</td>
<td>9.8</td>
</tr>
<tr>
<td>ER−</td>
<td>52</td>
<td>0.00</td>
<td>1.39</td>
</tr>
<tr>
<td>Total</td>
<td>134</td>
<td>3</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Fisher exact test, P = 0.0192.

N = number of samples included in the cohort; n and (%) = count and percentage of samples with alterations for the indicated site, respectively.
Because our cohort included 13 ER⁺ patient-matched primary-metastatic tumor pairs, we were able to explore if any metastatic lesions demonstrated an increase in CN as compared with their matched primary lesions. Indeed, we observed this for one pair, where the ER⁺ primary tumor BP51 had an ESR1 CN gain (CN 3.9), and the patient-matched brain metastases BM51 showed amplifications (CN 10.9; Fig. 2C and Additional file 1: Supplementary Fig. S3).

We have recently described a novel algorithm that helps identify potential gene fusions from hybrid capture DNA sequencing based upon CN exon imbalances at fusion breakpoints (18). The high resolution of nanoString exon-level CN detection within ESR1 allowed us to adapt this approach to determine ESR1 CN imbalance by calculating the ratio of CN signal of 5’ exon probes (3–6) to 3’ exon probes (7–10). Our analysis revealed that 5 of 11 amplified samples (45.5%) exhibited ≥30% increase in CN (ratio of ≥1.3) in the 5’ exons (Fig. 2D and Additional file 1: Supplementary Table S12, P = 0.0024), while no samples harbored an increase CN of 3’ exons relative to 5’ exons.

In summary, we observed enrichment of ESR1 amplifications in ER⁺, recurrent samples, and bone metastases versus other metastatic sites. We also identified frequent 5’–3’ exon imbalances indicating potential rearrangements in ESR1.

Correlation of ESR1 CN with ER mRNA expression

To determine whether ESR1 CN gains and amplifications were correlated with ER mRNA expression, we utilized expression data from RNA-seq. As expected, we observed higher ESR1 mRNA expression in samples with ESR1 gains/amplifications (Fig. 3A), although this association did not reach statistical significance, likely due to the limited number of samples in the CN gains/amplification group for which RNA-seq data were available (n = 3). An exploratory analysis within this group of ESR1-amplified samples showed a correlation between CN and expression, but this was also not significant (Additional file 2: Supplementary Fig. S2). The first translated ESR1 exon E3 was most predictive for mRNA expression (Fig. 3B and Additional file 1: Supplementary Table S13; rho = 0.34, P = 0.0219), which supports the higher CN on the 5’ side.

Validation of ESR1 CN amplifications enrichment in additional cohorts

To validate our findings of ESR1 CN amplifications, we queried three additional data sets (AACR-GENIE, MSKCC, and FM) that used sequencing to characterize breast cancer metastases (35, 36, 43, 44). This analysis revealed significant enrichment for ESR1 amplifications in metastatic versus primary tumors (Fig. 4A). The frequency of ESR1 amplifications was 1.65% (n = 27/1637), 2.28% (n = 19/835), and 1.84% (n = 122/6629) in the AACR-GENIE, MSKCC, and FM metastatic cohorts, respectively. Lower frequency of amplifications in these cohorts is likely due to the use of techniques less sensitive for measurement of amplifications.

In the FM cohort, where we have had access to ER status for a subset of the tumors, we observed a significant enrichment of ESR1 amplifications in ER⁺ (36/1272; 2.8%) compared with ER− samples (3/963; 0.3%; P = 1.9e-06). Analysis of survival data from MSKCC cohort showed significantly worse overall survival (OS) for patients with ESR1-amplified versus nonamplified tumors (P-value < 0.05, Fig. 4B), pointing toward potential clinical relevance of the amplification.

CN alterations of known and potential breast cancer driver genes other than ESR1

In addition to ESR1, we extended our CN analysis to 66 genes with described roles in breast cancer progression and metastasis. For these genes, we had slightly lower resolution due to lower coverage (2–3 probes per gene) compared with the ESR1 study (10 probes), which did not allow for high confidence detection of low-level amplifications, and thus we limited the analysis to binary calls (yes or no CNV, defined as CN ≥ 5). Across all ER⁺ and ER− tumors, the genes with most frequent CN amplifications are shown in Table 3 and Additional file 2: Supplementary Fig. S4. The most amplified genes were ERBB2 and GRB7, harboring amplifications of 44% and 21%, respectively, in brain and bone metastases, respectively. Previous studies have reported coamplifications of ERBB2 and GRB7 on amplicon 17q12 (45–47). This was confirmed by our eSNP-karyotyping analysis using RNA-seq data from a subset of our samples that predicts the amplification to cover a broader region rather than being limited to a focal event (Additional file 2: Supplementary Fig. S5). Metastatic site-unique or enriched CN alterations were seen for a number of genes, including higher rates of FADD amplifications (17%) in bone metastasis compared with other metastatic sites, and higher rates of PTK2 and PK14 amplifications (~10%–20%) in brain and GI metastases. Additionally, comparison of ER⁺ versus ER−
Brain metastases showed significant enrichment of FGFR1 amplifications in the ER+ group (Additional file 2: Supplementary Fig. S6; \( P = 0.0221 \)). The comparative analysis between ER+ and ER-/C0 tumors was only possible in brain metastases cohort where we had balanced distribution of ER+ versus ER-/C0 tumors. The most frequently deleted gene was TP53 (10%), and this loss was mainly observed in brain metastases (15.4%; Table 3 and Additional file 2: Supplementary Fig. S4).

Co-occurrence analysis identified gene combinations that are enriched in ER+ tumors (Fig. 5A, top; \( P < 0.05 \)). For example, we confirmed previously reported coamplifications of genes (CCND1, CTTN, FADD, PAK1, AAMDC, and FGF19) at the 11q13 amplicon (48–53), of MYC (8q24) and ERBB2 (17q12; refs. 54–56), and of NCOA3 (20q13) and MDM2 (12q15; ref. 57; Fig. 5B). We also observed coamplification of MDM2 and ERBB3 (12q13), which had not been previously described. Finally, there was an enrichment of MYC (45%) and CCND1 (36%) in tumors with ESR1 amplifications (Fig. 5C; \( P < 0.05 \)). We identified only one event of co-occurrence in ER-/C0 tumors; this involved CN amplifications of ERBB2 and GRB7 at the 17q12 amplicon (Fig. 5A, bottom).

Among the most frequent recurrent deletions, we identified mutually exclusive deletions of CDKN2A, CDKN2B, and CDKN1B with amplifications of CCND1 and CDK4/6 (Fig. 5D; \( P < 0.0001 \)). Expression analysis from RNA-seq data showed significant correlation with these CN alterations (Fig. 5D).

### Discussion

Breast cancer is increasingly being recognized as a disease that can be driven by DNA structural variation, in addition to well-described single base-pair mutations. The majority of CN studies

### Table 3. Most frequent CN alterations by site

<table>
<thead>
<tr>
<th>Alteration</th>
<th>Gene</th>
<th>Primary</th>
<th>All Mets</th>
<th>Brain</th>
<th>Bone</th>
<th>Ovary</th>
<th>GI</th>
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<td>Amplifications</td>
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<td>31 (28.7)</td>
<td>23 (44.2)</td>
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<td>2 (8.6)</td>
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<td></td>
<td>GRB7</td>
<td>9 (34.6)</td>
<td>29 (26.8)</td>
<td>24 (46.1)</td>
<td>2 (8.3)</td>
<td>2 (8.6)</td>
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<td></td>
<td>CCND1</td>
<td>1 (3.8)</td>
<td>13 (12)</td>
<td>6 (11.5)</td>
<td>5 (20.8)</td>
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<td></td>
<td>MYC</td>
<td>4 (15.3)</td>
<td>13 (12)</td>
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<td>FGF19</td>
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\( n \) and (%) = count and percentage of samples with alterations for the indicated site, respectively.
have been performed in primary tumors, at least in part due to difficulties in accessing metastatic and recurrent tissue. However, it is now well recognized that expression of key biomarkers such as ER and HER2 can change between a primary and MBC (58), and that this has clinical significance for therapeutic decision-making.

Recent studies have documented ESRI base-pair mutations and fusions in breast cancer. ESRI amplification has also been identified, although there have been conflicting reports on the frequency, ranging from 0% to 30% (3–8). CN has been explored using FISH, aCGH, and qPCR, and sequencing is currently evolving as promising approach. Due to the potential clinical relevance, we performed a comprehensive measurement of ESRI CN (by examining all exons) in a unique cohort of metastatic, and when available patient-matched primary tissues.

Figure 5. Co-occurrence and mutual exclusivity of CN alterations in MBC. A, Tile plots of ER$^+$ (top) and ER$^-$ (bottom) samples for CN amplifications co-occurrences. Gene pairs with significant co-occurrence are colored in red and marked with *** in the plot (multiple Fisher exact test with FDR-adjusted P < 0.05). B, Circos plot for genomic co-occurrence events. Circular tracks from outside to inside: genome positions by chromosomes (black lines are cytobands); inside arcs connect genes with co-occurrence (red, interchromosomal; blue, intrachromosomal). C, Oncoprint shows enrichment of MYC (45%) and CCND1 (36%) amplifications in tumors with ESRI amplifications (Fisher exact test; MYC P = 0.0083, CCND1, P = 0.0365). D, Top: Oncoprint of samples with CN alterations in the CDKs pathway and its inhibitors. Amplifications of the activators CCND1 and CDK4/6 of the pathway were mutually exclusive to the deletions of the inhibitory members CDKN1B, CDKN2A, and CDKN2B (Fisher exact test P < 0.0001). Bottom: RNA-seq data for a subset of samples (n = 46) show significant correlation of mRNA expression with CN status for genes of interest (P indicated for Wilcoxon rank-sum test).
In total, we detected ESR1 gene gains or amplifications in 13% of ER+ metastatic breast tumors. Given the sensitivity and specificity of our method, and careful sampling of tumors with cellularity of at least 40%, we are confident that this is a representative number for ER+ metastatic tumors. Previously reported wide ranges in detected CN alterations are due, at least in part, to the use of technologies with limited sensitivities and specificity, such as aCGH, FISH, and qPCR. Our study clearly shows PCR approaches using a limited number of reference genes need to be interpreted with caution. Additional reasons for variability include tumor cellularity and threshold for calling gain and amplification, as previously discussed (39).

We observed significant enrichment of ESR1 gains and amplification in ER+ metastatic lesions compared with the primary tumors, and we were able to validate this finding in three large data sets. In 1 of 15 ER+ paired samples, ESR1 CN increased from 3.9 in the primary tumor to 10.9 in the patient-matched brain metastasis. This was not due to cellularity, because both the primary tumor and the metastatic lesion for sample #51 had similar tumor cellularities (~70%). In support of our findings, a recent study by Ullah and colleagues showed an ESR1 amplification in an ER+ metastatic lesion, but not in the paired primary tumor (60). This enrichment suggests selection of ESR1-amplified clones under endocrine treatment, similar to that described for ESR1 mutations (25, 26) and HER2 amplifications in brain metastases (16). Although somewhat limited by small numbers, we did observe a correlation between ESR1 amplifications and ESR1 mRNA expression, a finding that has previously been reported by others (2, 49). However, there are reports in which ESR1 amplification does not correlate with expression level (5, 59, 62), and ESR1 amplifications have been previously detected in ER- tumors with poor prognosis (63). Of note, in our study, we observed an ESR1 gain (CN = 2.7) in only one ER- sample. Additional studies are necessary to decipher the functional consequences of low-level ESR1 amplifications.

We (18) and others (17, 64) have recently identified ESR1 gene fusions in MBC, with fusions generally maintaining the DNA binding and N-terminal transcriptional activation domains, but deleting the ligand-binding domain. We recently described a copyshift algorithm that determines imbalance in DNA CN of exons that are 5 or 3 to the break (18). The high resolution of our nanoStringing assay allowed us to detect CN imbalances at the exon level, and we found that 5 of 11 amplified samples (45.5%) showed at least 30% increase in CN at the 5' side of ESR1. We did not have RNA-seq data for 4 of these 5 cases, and we were unable to detect an ESR1 fusion in the sample with available RNA-seq data, likely due to inadequate sequencing coverage. Other mechanisms for generation of structural variants could be involved, such as fusions resulting from tandem duplications. An example is the ESR1–CCDC170 fusion (65) in which CCDC170 is truncated but the coding region of ESR1 is intact. Overall, these imbalances are indicative of genetic rearrangements, therefore, future studies should determine whether tumors with significant exon imbalances harbor ESR1 fusion genes, and if so, which ones might be nonfunctional versus drivers of endocrine resistance.

The clinical relevance of ESR1 CN is unclear at this point in time. ESR1 amplifications have been associated with improved (2, 59, 61) as well as worse outcome (66) in patients treated with endocrine therapy. In endocrine-resistant cell line models (67) and in a PDX model (17) with ESR1 amplifications, estradiol treatment resulted in tumor regression. We recently described an MBC case with ESR1 amplifications that showed sustained partial response to high-dose estradiol treatment as measured by CA 27–29 level and by decrease in liver metastasis burden (68). These findings might deserve further exploration in a clinical trial setting including prospective measurement of ESR1 CN.

Analysis of 66 other genes revealed frequent ERBB2 and GRB7 amplifications in different metastatic sites, with an enrichment in brain metastases. These results support the increasingly growing need for testing HER2 in the metastatic settings (69, 70). Amplification of genes at the 11q13 locus has been reported in about 15% of primary breast cancer cases and is associated with poor prognosis (48–53). In our analysis, amplifications of multiple genes at this locus (CCND1, CTNN, FADD, PAK1, AAMDC, and FGFR4) were also frequent in multiple metastatic sites. Intriguingly, we observed coamplification of ERBB3 and MDM2, which has not been previously described. Given prior evidence for functional interaction between ERBB3 signaling and MDM2 complex formation, coamplification may be positively selected in some tumors. Similar to ESR1, some amplifications also showed organ-specific enrichments, suggesting CN alterations may be driving metastatic tropisms. For example, brain and GI metastases showed higher PTK2 and PKIA amplifications (~10%–20%), while FADD amplifications were more frequent in bone metastases (17%). In our patient-matched samples, most of the CN alterations were maintained in the tumors except for two pairs with a slight increase in CN for PKIA, PTK2, and FGFR4 in the metastases (Additional file 2: Supplementary Fig. S7). Moreover, FGFR1 amplifications were enriched in ER+ versus ER- brain metastases.

Cyclin-dependent kinases (CDK), which control transitions through the different stages of the cell cycle, have been considered as promising targets for cancer therapy. Amplifications/overexpression of CCND1 (CDK4/6 activator) and loss of CDKN2A (p16, CDK4/6 inhibitor) have been described in primary breast cancer patients (71–77). Preclinical and in vitro data across multiple cancers support that loss of CDK4/6-inhibitory members may serve as biomarkers for CDK4/6 inhibition sensitivity (78–83). On the other hand, clinical evidence in breast cancer trials performed thus far showed that CCND1 amplifications or p16 loss is unlikely to predict treatment benefit (84). However, these trials assessing p16 as a biomarker were earlier phase with small sample size, and did not show if concurrent alterations in other genes existed, which can alter response to therapy. In our analysis, 32 of 108 metastases (29%) showed aberrations in the CDKs pathway and 24 samples (22%) had alterations in the CCND1–CDK4–CDK6 axis specifically. Among those, deletions of the CDK4/6 inhibitors CDKN2A/B were significantly mutually exclusive with CCND1 amplifications, and correlated with mRNA expression, suggesting functional consequences for the alterations. Those deletions were maintained in the paired metastases, which supports the concept of using CDK4/6 inhibitors in metastatic settings as previously shown (85–88). Moreover, it is important to note that these deletions also occur in ER- tumors and tumors with HER2 amplifications. Most clinical trials have been conducted in patients with ER+/HER2- breast cancer. Our data suggest that testing of CDK4/6 inhibitors in other subgroups (e.g., ER-) may deserve further investigation.
Conclusion

Our findings suggest that, in addition to ESR1 mutations, ESR1 amplifications and exon imbalances might play a role in endocrine resistance, but further studies are necessary to test this hypothesis. Our analysis also defines CDK pathway alterations as common in metastatic disease—which could be especially relevant to recently adopted CDK4/6 inhibitor therapy. Taken together, CN alterations in druggable genes are common in MBC and could serve as biomarkers for precision therapies in advanced disease.

Ethics approval and consent to participate

Collection and analysis of specimens were approved under the University of Pittsburgh IRB # PRO 11100645, 14040193, 1500502, 16020301, and Charité Universitätsmedizin Berlin guidelines.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Disclosure of Potential Conflicts of Interest

R.J. Hartmaier has ownership interest (including stock, patents, etc.) in A. Basudan, A. Bahreini, R.J. Hartmaier, R.J. Watters, P.C. Lucas, A.V. Lee, S. Oesterreich

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